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INCREASING THE STABILITY OF ANTAGONISTIC FUNGUS, TALAROMYCES FLAVUS, USING SOME ORGANIC AND MINERAL COMPOUNDS

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ABSTRACT

The important issues of interest to the biological fungicides production companies are marketing capabilities and commercialization of these compounds. According to the investigations made, increasing the efficiency and sustainability of such compounds, commercialization of important factors affecting the marketing capabilities and considered them. In this study, for increasing the stability of bioformulations related to different Talaromyces flavus isolates, the most effective stabilizers related to its metabolites (kitinase enzyme, glucose oxidase enzyme and ethilen compounds) were used. Based on previous research, the most effective platforms in terms of sporulation and stability depending on T. flavus isolates, rice bran for the isolates related to the potato fields (TF-Po-V-48), sugar beet fields (TF-Su-K-1) and cotton fields (TF-Co-G-1) and peat soil mixed with rice bran for the isolates related to cultivated areas related to tomato (TF-to-V-29 and TF-to-U-38) and greenhouse cucumber (TF-Cu-V-59) was introduced. So, this research was performed in two separate experiments, for substrates peat soil mixed with rice bran and rice bran corresponding to a completely randomized design in fifteen treatments and three replications. The treatments consisted of a combination of three different T. flavus isolates with each of the five used stabilizers including aminophenol, dicycloserine, carboxymethyl cellulose, magnesium sulfate and sodium nitrate. Based on the stability of T. flavus isolates, the treatments were evaluated as determining the percent of active ascospores. The evaluation time range, three weeks after treatment developing was started and every 90 days for 21 months continued. The results showed that for all treatments, the highest level of stability in the second quarter happened. In both experiments, peat soil mixed with rice bran and rice bran, dicycloserine, sodium nitrate and magnesium sulfate were very efficient in increasing the the stability of different T. flavus isolates, when compared to two other stabilizers (aminophenol and carboxymethyl cellulose). Among these stabilizers, sodium nitrate for the isolates related to sugar beet and dicycloserine for other isolates caused the maximum percentage of T. flavus active ascospores.

KEYWORDS: Non-Volatile Compound, Talaromyces Flavus, Stability & Volatile Compound

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Original Article

INTRODUCTION

Biological control using fungal and bacterial antagonists in recent years, have been applied to control plant important diseases (Ardekani *et al*., 2009; Shahraki *et al*., 2009; Heydari and Pessarakli, 2010; Kakvan *et al*., 2013; mansoori *et al*., 2013). Regarding the importance of soil diseases such as Verticillium wilt, Fusarium wilt and seedling death in various crops such as cotton, potato, tomato and greenhouse cucumber in Iran (Naraghi *et al*., 2010a, 2010b, 2010c, 2012a, 2012b, 2014a, 2014b) and mention the fungus *Talaromyces flavus* as an effective

antagonist against the major causes of the above diseases including *Fusarium oxysporum*, *Verticillium albo-atrum*, *Verticillium dahliae* and *Rhizoctonia solani* in foreign scientific sources (Madi *et al*. 1992; Madi *et al*., 1997; Duo-Chuan *et al*., 2005; Haggag *et al*., 2006; Shakhul Ashraf and Ahmad Khan, 2007), also in Iran, different isolates of this antagonist fungus were taken from the main regions of cultivation of some crops (Naraghi *et al*., 2010a, 2010b, 2010c, 2014a, 2014b).

After several laboratory, greenhouse and field investigations, in order to determine the antagonistic effects of these isolates against these pathogenic agents, and introduce the most effective isolates in terms of controlling the pathogens of each crop, biological fungicides containing different *T. flavus* isolates and other antagonistic fungal and bacterial isolates were prepared separately to control the pathogenic agents of each crop (Naraghi *et al*., 2014a and b; Farhang Niya *et al*. 2015). For the application of these fungicides on a wide scale, there is a need for their mass production and the transfer of the technical knowledge of fungicide production to the producing companies. In this regard, important issues of interest to such companies can be marketing capability and commercialization of fungicide (Alimi *et al*., 2006; Husen *et al*., 2007; Kaewchai *et al*., 2009; Pereira *et al*. 2009). According to the research conducted, increasing the efficiency and stability of these compounds is one of the important factors influencing marketing capability and commercialization (Kaewchai *et al*., 2009; Mukhopadhyay and Maiti, 2009; Ghaderi-Daneshmand *et al*., 2012).

In this study, according to the previous studies on the determination of the dominant antagonistic mechanisms of *T. flavus* isolates for some pathogens in these products and the identification of specific metabolites of mechanisms in existing scientific sources, using the research carried out outside the country on the basis of the introduction of stabilizing organic compounds for metabolites (Yu and chang, 1987; Cimarelli *et al*. 2001; Matos *et al*., 2012), the substrate of these isolates can be optimized to enhance their efficacy through increasing *T. flavus* metabolite's durability. On the other hand, due to the effect of these chemical compounds on the growth rate of spores of fungi, including antagonistic fungi such as *Beauveria bassiana* (Gao, 2011), among the stabilizers studied, the most effective ones should be selected to increase the stability or percentage of active spores of *T. flavus*. Thus, with the preliminary studies in the field of this research and its implementation, the following goals are realized:

Different groups of organic compounds will be identified to increase the durability of the metabolites derived from *T. flavus*.

Among the compounds in each group, the most effective ones are introduced in terms of increasing *T. flavus* stability.

MATERIALS AND METHODS

Determine the Stabilizing Compounds to be used in *T. flavus* Bioformulation to Increase their Efficacy

According to the previous studies (Naraghi *et al*., 2010a, 2010b and 2010c), *T. flavus* isolates used in six *T. flavus* bioformulations and their inhibitory mechanisms to be used against major soil diseases of crops such as sugar beet, cotton, potatoe, tomatoe and cucumber are listed in Table 1. Therefore, according to the metabolites obtained from various inhibitory mechanisms (mycoplasm, production of volatile compounds and the production of non-volatile compounds), according to the sources mentioned in Table 2, the stabilizers of *T. flavus* metabolites were identified.

Table 1: The Most Effective Isolates of Talaromyces Flavus and their Inhibitory Mechanisms for Application against Major Soil Diseases of the Greenhouse Products of Cotton, Potato, Tomatoe and Cucumber

SI. No	T. Flavus Isolate	Inhibitory Mechanism	Target Soil Disease	Relevant Crop Product
1	TF-Co-G-1 From Gorgan cotton field	Produce volatile compounds	Verticillium wilt and Rhizoctonium seedlings death	Cotton
2	TF-Su-K-1 From Karaj sugar beet field	Produce volatile compounds	Rhizoctonium and Fusarium seedlings death	Sugar beet
3	TF-Po-V-48 From Varamin potato field	Produce volatile compounds	Verticillium wilt	Potato
4	TF-To-V-29 From Varamin tomato field	Mycoparasitism	Fusarium wilt	Tomato
5	TF-To-U-36 From Urmia tomato field	Produce volatile compounds	Verticillium wilt	Tomato
6	TF-Cu-V-59 From Varamin cucumber greenhouse	Produce volatile compounds	Verticillium wilt	Greenhouse cucumber

Table 2: Stabilizers of Metabolites Related to Inhibitory Mechanisms of different Isolates of Talaromyces Flavus

SI. No	Inhibitory Mechanisms of different Isolates of T. Flavus	Metabolites Related to Inhibitory Mechanisms	Stabilizers of Metabolites
1	Mycoparasitism	Chitinase enzyme Inbar and Chet, 1995	Osmotic stabilizers such as sodium nitrate and magnesium sulfate Yu and Chang, 1987
2	Produce volatile compounds	Glucose oxidase, beta-galactosidase and giziolidase enzymes Ward, 2011	Dyscyclocerin and carboxymethylcellulose compounds Matos *et al*., 2012
3	Produce volatile compounds	Ethylene, Hydrogen, Cyanide, Alcohol and Aldehyde Compounds Keszler *et al*., 2000	Amino-phenyl compounds Cimarelli *et al*., 2001

Determine the Most Effective Stabilizer for Increasing the Stability of different Strains of T. Flavus

This study was conducted in two separate experiments for the substrate of rice bran and peat soil together with rice bran, using appropriate isolates in a completely randomized design with 15 treatments and three replications.

The experiment treatments using rice bran included:

Separate compounds of three different *T. flavus* isolate (TF-Co-G-1, TF-Po-V-48 and TF-Su-K-1) with each of the five stabilizers used including cycloserine, carboxymethyl cellulose,

Sodium nitrate, magnesium sulfate and aminophenol

The experiment treatments using peat soil with rice bran included:

Separate compounds of three different *T. flavus* isolates (TF-To-U-36, TF-To-V-29 and TF-Cu-V-59) with each

of the five stabilizers used including cycloserine, carboxymethyl cellulose, sodium nitrate, magnesium sulfate and aminophenol. Assessing the stability of *T. flavus* isolates was as determining the percentage of active *T. flavus* acospores as follows:

First, different isolates of *T. flavus* were cultured on the substrate of rice bran for cotton, potato and sugar beet isolates (Naraghi *et al*., 2010a and Naraghi *et al*., 2012a) and peat substrate with rice bran with a ratio of 3:2 for the greenhouse tomato and cucumber isolates (Naraghi *et al*., 2010b and c). In this way, different *T. flavus* isolates were cultured on specific TF Medium including one liter of distilled water, 39 g of commercial PDA, 2 ml of 50% lactic acid solution, 100 mg of streptomycin sulfate, 50 mg of chloro tetracycline, 50 milligram of chloramphenicol, 40 mg of primarysin as 2.5% suspension, 30 mg nisatin and 0.5 g of x gal (Marois *et al*., 1984) and kept for 3 weeks to produce ascospore at 30 °C. Then, adding 10 ml of sterilized distilled water to each petri dish and rinsing the ascospores produced, the suspension with a concentration of 10^6 ascospores per ml of sterilized distilled water was prepared and 10 ml was added to each of the cellophane bags containing 50 g of used substrate that was previously autoclaved and mixed completely. The inoculated bags were placed at 30 °C for 3 weeks until fungal ascospores were completely observed on the substrate surface. After three weeks, the content of the cellophane bags was removed and completely dried at laboratory temperature (about 25 °C) (Naraghi *et al*., 2010a).

Then, the stabilizing compounds were added to each substrate according to the treatment and the amount of adding the supplements to the culture media (10 ml of the supplement solution at 20 g / l for 250 g of each substrate) (Engelelkes *et al*. 1997). It should be noted that in the present study, additive supplements were made up of solutions containing stabilizing compounds. At the next stage, the substrates were kept at laboratory temperature. The time to determine the percentage of active spores started at 3 weeks after culturing and continued at intervals of thre months (90 days) until the end of the 21st month (the 7th three months (quarterly)) (Naraghi *et al*., 2010a).

To carry out this stage of the study, one gram of each of the bioformulations prepared for each isolate was added to 9 milliliters of sterilized distilled water, and the ascospores contained in one milliliter of the suspension were counted by a homocytometric slider. Then, the suspension of 10 ascospores per ml was prepared and, according to Marois *et al*. (1984) method, one milliliter of the suspension prepared was put on the surface of each of the three petri dishes containing the selected culture medium of *T. flavus* (Marois *et al*., 1984), which was intended for each replication. Petri dishes were kept in the incubator at 30 °C for three to five days. By observing yellow colonies on the surface of petri dishes, the percentage of active *T. flavus* oscospores in each replication was determined by calculating the average percentage of spores in three petri dishes. It should be noted that at this stage, according to the previous experiences, the suspension was passed through sterile cotton to prepare spore-containing suspension and remove mycelium components, also the spores were examined under a microscope to ensure the growth. At the next stage, the data obtained from each of the two experiments of rice bran and peat soil with rice bran were separately analyzed using a completely randomized design with software program MSTAT-C. Means and compared with Duncan's multiple range tests.

RESULTS

Determine the Most Effective Stabilizer for Increasing the Stability of different Strains of T. Flavus

A. The Experiment using Rice Bran

Determine the Percentage of Active or Inactive T. Flavus Spores for each Bioformulation

The experiment of the effect of *T. flavus* bioformulations containing rice bran and different stabilizers was significant on the percentage of active *T. flavus* spores from the first to the third three months at 1% probability level. The results showed that in the period the highest percentage of active T. flavus spores occurred in the second three months, and this level has been reduced to the 7th three months after that (Table 3).

According to the statistical grouping, from the first to the seventh three months, all the treatments were in six, five, seven, six, five, seven, and eight statistical groups, respectively (Table 3). In terms of assessment time, the highest percentage of active spores was for the treatments of TF-Su-K-1 with nitrate-sodium stabilizer, TF-Co-G-1 and dicycloserin stabilizer, TF-Po -V-48 with dicycloserin stabilizer and TF-Po-V-48 with sodium nitrate stabilizer, while the lowest percentage of active spores was for the treatments of TF-Su-K-1 with aminophenol stabilizer and TF-Co-G-1 with each of aminophenol and carboxymethylcellulose stabilizers (Table 3).

Table 3: Grouping the Percentage of Active Talaromyces flavus Spores in the Experiment of Bioformulations Prepared by Rice Bran from the First Three Months - Three Weeks to the Seventh Three Months after Culturing

Treatment	Mean Percentage of Active Spores (%) *						
	The 1st Three Months	The 2nd Three Months	The 3rd Three Months	The 4th Three Months	The 5th Three Months	The 6th Three Months	The 7th Three Months
TF-Su-K-1-Aminophenol	60.00c	50.00e	46.66e	43.33e	40.00d	36.66e	33.33f
TF-Su-K-1-Dyscyclocerin	100.00a	90.00b	86.66ab	83.33ab	80.00ab	76.66ab	73.33ab
TF-Su-K-1-Carboxymethylcellulose	40.00d	70.00d	66.66d	63.33d	60.00c	56.66d	53.33de
TF-Su-K-1-Magnesium sulfate	40.00d	80.00c	76.66bcd	76.66bc	73.33b	70.00bc	66.66bcd
TF-Su-K-1-Sodium Nitrate	30.00e	100.00a	96.66a	93.33a	90.00a	86.66a	83.33a
TF-Co-G-1-Aminophenol	40.00d	53.33e	50.00e	46.66e	43.33d	40.00e	33.33f
TF-Co-G-1-Dyscyclocerin	86.66b	100.00a	96.66a	93.33a	90.00a	86.66a	83.33a
TF-Co-G-1-Carboxymethylcellulose	23.33f	50.00e	46.66e	43.33e	40.00d	36.66e	30.00f
TF-Co-G-1 Magnesium Sulphate	23.33f	73.33d	70.00cd	66.66cd	63.33c	60.00cd	56.66cde
TF-Co-G-1 Sodium Nitrate	20.00f	86.66b	83.33abc	80.00ab	76.66b	73.33b	70.00abc
TF-Po-V-48-Aminophenol	60.00c	70.00d	66.66d	63.33d	60.00c	56.66d	50.00e
TF-Po-V-48-Dyscyclocerin	100.00a	100.00a	96.66a	93.33a	90.00a	86.66a	83.33a
TF-Po-V-48-Carboxymethylcellulose	40.00d	70.00d	66.66d	63.33d	60.00c	56.66d	50.00e
TF-Po-V-48-magnesium sulfate	40.00d	86.66b	83.33abc	80.00ab	76.66b	73.33b	70.00abc

Table 3: Contd.,							
TF-Po-V-48-Sodium Nitrate	30.00e	100.00a	96.66a	93.33a	90.00a	86.66a	83.33a

* No significant difference between the treatments with similar letters at 1% probability level.

B) Experiment using Peat Soil with Rice Bran

Determine the Percentage of Active or Inactive T. flavus Spores for each Bioformulation

The experiment of the effect of *T. flavus* bioformulations containing rice bran and different stabilizers was significant on the percentage of *T. flavus* active spores from the first to the third three months at 1% probability level. The results of this experiment using peat soil substrate with rice bran as the experiment of rice bran substrate showed that in this period the highest percentage of active *T. flavus* spores occurred in the second three months and this amount has been reduced to the seventh three months after that (Table 4).

According to the statistical grouping, from the first to the seventh three months, all the treatments were in six, four, two, six, two, and four groups, respectively (Table 5). During the mentioned period, the highest percentage of active spores belonged to the treatments containing dyscyclocerin, magnesium sulfate and sodium nitrate stabilizers (Table 5). In the evaluation period, the lowest percentage of active *T. flavus* spores belonged to treatments containing aminophenol and carboxymethyl cellulose (Table 4).

Table 4: Grouping the Percentage of Active Talaromyces Flavus Spores in the Experiment of Bioformulations Prepared by Peat Soil Substrate with Rice Bran from the First Three Months - Three Weeks to the Seventh Three Months after Culturing

Treatment	Mean Percentage of Active Spores (%) *						
	The 1st Three Months	The 2nd Three Months	The 3rd Three Months	The 4th Three Months	The 5th Three Months	The 6th Three Months	The 7th Three Months
TF-To-V-29-Aminophenol	20.00cd	40.00c	36.66b	33.33e	30.00b	26.66b	02.00c
TF-To-V-29-Dyscyclocerin	50.00a	86.66a	83.33a	80.00ab	76.66a	73.33a	07.00a
TF-To-V-29-Carboxymethylcellulose	16.66de	40.00c	36.66b	33.33d	30.00b	26.66b	02.00c
TF-To-V-29-Magnesium sulfate	26.66cd	80.00b	76.66a	73.33bc	70.00a	66.66a	06.00b
TF-To-V-29- Sodium Nitrate	20.00cd	76.66b	73.33a	70.00a	66.66a	63.33a	06.00b
TF-To-V-36-Aminophenol	20.00cd	40.00c	36.66b	33.33e	30.00b	26.66b	23.33c
TF-To-V-36-Dyscyclocerin	46.66a	86.66a	83.33a	80.00a	76.66a	73.33a	70.00a
TF-To-V-36-Carboxymethylcellulose	13.33e	36.66c	33.33b	30.00e	26.66b	23.33b	20.00c
TF-To-V-36-Magnesium sulfate	23.33bc	80.00b	76.66a	73.33cd	70.00a	66.66a	06.00b
TF-To-V-36- Sodium Nitrate	16.66de	76.66b	73.33a	70.00ab	66.66a	63.33a	06.00b
TF-Cu-V-59-Aminophenol	20.00cd	30.00d	26.66b	23.33d	20.00b	16.66b	10.00d
TF-Cu-V-59-Dyscyclocerine	46.66a	86.66a	83.33a	80.00a	76.66a	73.33a	07.00a

Table 4: Contd.,							
TF-Cu-V-59-Carboxymethylcellulose	16.66de	36.66c	33.33b	30.00d	26.66b	23.33b	02.00c
TF-Cu-V-59-Magnesium sulfate	26.66b	80.00b	76.66a	73.33ab	70.00a	66.66a	06.00b
TF-Cu-V-59- Sodium nitrate	20.00cd	80.00b	73.33a	70.00a	66.66a	63.33a	06.00b

* No significant difference was found between treatments with similar letters, at 1% probability level.

DISCUSSIONS

The results of this study showed that the stability rate (the percentage of active spores) and active population of *T. flavus* increased to the 3rd three months in the period from the first thre months to the seventh three months after the production of various isolates of *T. flavus*. The percentage of active spores for biofurmulation containing the isolate related to cotton was 96%; after this time to the 6th three months, this rate reduced by 10%. In another study on the stability of *T. flavus* on various plant residues without the use of chemical stabilizers, the highest stability was observed in the second three months (100%), while the reduction in the level of stability from the 3rd to 6th three months was estimated 50% (Naraghi *et al*., 2007). Therefore, it can be concluded that the reason for increasing the time associated with the highest level of stability and reducing the rate of stability until the 6th three months was the presence of *T. flavus* metabolites' stabilizers and, consequently, growth and sporulation of the fungus. In a study on the effect of different culture mediums on the biosynthesis of secondary metabolites of *Penicillium verrucesum*, it was found that the amount of fungal metabolites was effective on the growth and activity of this fungus (Elias *et al*., 2006).

Also, the results of this stage of the study showed that in most of *T. flavus* bioformulations, dyscyclocerin has caused increasing spores' stability or growth of *T. flavus* isolates. Dyscyclocerin is an amino acid derivative; therefore, this result was consistent with the results of the previous studies on the development of germination of spores of fungi, including antagonistic fungi such as T. flavus and B. bassiana, by nitrogen sources such as alpha-amino nitrogen (Engelkes *et al*., 1997; Gao and Xingzhong, 2010; Gao, 2011).

On the other hand, the results of this study showed that in most of *T. flavus* isolates, the combination of magnesium sulfate was also effective on increasing the percentage of active spores. In this regard, Kim *et al*. (1988) showed that the presence of sulfate magnesium plays a major role in the production of volatile and non-volatile metabolites of *T. flavus*, and these metabolites have also contributed to the fungus spores' growth and development (Calvo *et al*., 2002).

Also, according to the assessment results in the first and the second three months, *T. flavus* stability trend in bioformulations containing various chemical stabilizers showed that some stabilizers such as sodium nitrate salt and/ or magnesium sulfate in the first three months, in terms of efficacy, did not increase the level of *T. flavus* stability to dyscyclocerin, but from the second three months, the bioformulations containing these salts with dyscyclocerin content in terms of the percentage of active *T. flavus* spores were in a statistical group. Many studies have shown that some environmental factors such as soil acidity and/ or organic salts, such as sodium nitrate and magnesium sulfate, were initially considered as stress for growth of the fungus spores, but after three months, due to the fungus adaptive reaction, the presence of such compounds has not had a negative impact on the fungus growth and activity (Rodriguez *et al*., 2008; Shanmugam *et al*., 2010; Hiscox *et al*., 2015).

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